Phytotoxicity, cytotoxicity and genotoxicity evaluation of organic and inorganic pollutants rich tannery wastewater from a Common Effluent Treatment Plant (CETP) in Unnao district, India using *Vigna radiata* and *Allium cepa*

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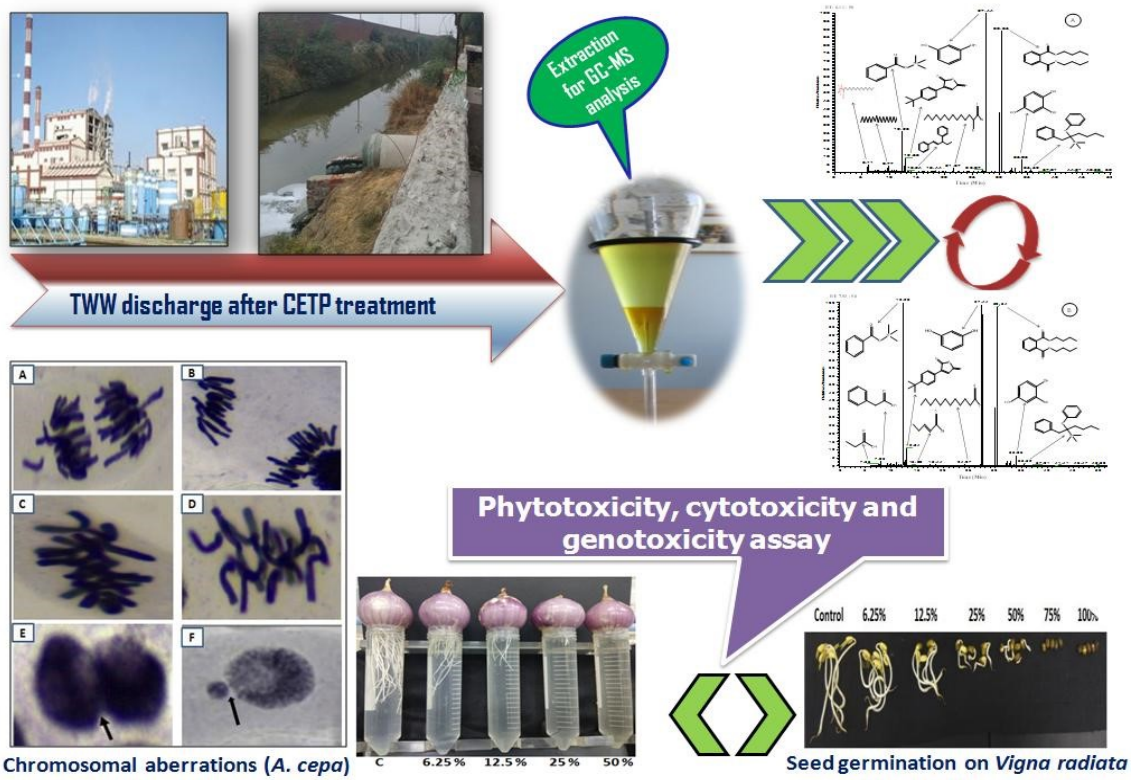
Graphical abstract



Abstract

The leather industry is a major source of environmental pollution in India. The wastewater generated by leather industries contains very high pollution parameters due to the presence of a complex mixture of organic and inorganic pollutants even after the treatment at a Common Effluent Treatment Plant (CETP) and disturbs the ecological flora and fauna. The nature, characteristics and toxicity of CETP treated wastewater is yet to be fully elucidated. Thus, this study aims to characterize and evaluate the toxicity of CETP treated tannery wastewater collected from the Unnao district of Uttar Pradesh, India. In addition to measuring the physico-chemical parameters, the residual organic pollutants was identified by GC-MS analysis and phytotoxicity, cytotoxicity and genotoxicity of the treated wastewater was evaluated using *Vigna radiata* L. and *Allium cepa* L. Results showed that the treated wastewater contained very high pollution parameters (TDS 3850mg/L, BOD 680mg/L, COD-1300mg/L). GC-MS analysis revealed the presence of various types of residual organic pollutants including benzoic acid, 3-[4,-(T-butyl) Phenyl] furan-2-5-dione, benzeneacetamide, resorcinol, dibutyl phthalate, and benzene-1,2,4-triol. Further, toxicological studies showed the phytotoxic nature of the wastewater as it inhibited seed germination in *V. radiata* L. and root growth of *A. cepa*. Genotoxicity was evidenced in the root tip cell of *A. cepa* where chromosomal aberrations (stickiness, chromosome loss, C-mitosis, and vagrant chromosome) and nuclear abnormalities like micronucleated and binucleated cells were observed. Thus, results suggested that it is not safe to discharge these wastewater into the environment.

Keywords: Tannery wastewater; Residual organic pollutants; Genotoxicity; Chromosomal aberrations

1. Introduction

The leather industry is an important economic sector in many developing countries including India. However, it is also a major source of environmental pollution due to the discharge of huge volume of potentially toxic and hazardous wastewater into the receiving water body, which negatively affect societies (Dixit et al., 2015; Montalvão et al., 2017; Chowdhary et al., 2018). The wastewater discharged from leather industries are characterized by high pH, chemical oxygen demand (COD), biochemical oxygen demand (BOD), total dissolved solids (TDS), chromium, sulfate, phosphate, chloride and highly toxic organic pollutants that makes the wastewater unfit for irrigation and poses serious damage to plants and human being (Kumari et al., 2016; Bharagava and Mishra, 2018). In India, there are more than 2500 tanneries, of these, nearly 80% are based on chrome tanning process which account for 15% of the total worldwide leather production (Shukla et al., 2009; Chandra et al., 2011). The majority of small-scale tanneries cannot afford their own effluent treatment plant, instead they depends on a central facility, the Common Effluent Treatment Plant (CETP), to manage their wastewater. In the CETP, combined effluent from nearby tanneries are brought to a central place for the treatment (Pathe et al., 2004). More than 150 have been set-up so far under the Indian government scheme for the treatment of industrial wastewater.

The continuous discharge of the tannery wastewater into the environment is of serious eco-toxicological concerns (Matsumoto et al., 2006; Bharagava and Mishra, 2018). The chemicals used in tannery for the tanning process include synthetic organic pollutants like tannins, pthalates, phenolic compounds, azo dyes, surface-active compounds, pesticides, sulphonated oils and grease that are not completely degraded through secondary CETP and are released untreated. Continuous releases of residual organic pollutants in tannery wastewater into the Ganga River through the drains have been a growing environmental concern (Tare et al., 2003; Alam et al., 2009; Chandra et al., 2009) and require urgent attention for the protection of environment and human health. The nature and characteristics of the

residual organic pollutants in tannery wastewater, which are not significantly degrade during the secondary treatment process at CETP, have yet to be fully investigated.

The present study aims to characterize and identify the residual organic pollutants remained in tannery wastewater after the secondary treatment process carried out at a CETP in the Unnao district of Uttar Pradesh, India and to evaluate the phytotoxicity, cytotoxicity and genotoxicity assessment of these residual organic pollutants present in tannery wastewater using agriculture crop *Vigna radiata* and *Allium cepa*.

A. cepa L. has been regarded as a suitable plant model to assess chromosomal damage and disturbances in the mitotic cycle due to the presence of good chromosome conditions such as large chromosomes and in a reduced number (2n=16). The *A. cepa* test, a relatively easy, rapid, sensitive and highly reproducible plant model has been strongly recommended for the toxicity/genotoxicity evaluation of environmental contaminants present in water, wastewater, sludge and soils (Fiskesjo, 1985; Leme and Marin-Morales, 2009; Haq et al., 2017).

2. Materials and Methods

2.1. Collection of treated tannery wastewater and its physico-chemical characterization

The wastewater samples collected from the outlet of CETP-Unnao, located in the Unnao district of Uttar Pradesh, India (Fig. 1) in pre-sterilized plastic containers (capacity 5-L) were brought to the laboratory and stored at 4 °C. CETP-Unnao was in operation since 1994. This is an activated sludge process (ASP) based CETP, treating ~1.9 MLD wastewater received from a cluster of ~25 tanneries located in nearby areas against a design flow of ~2.15 MLD. The quality of the treated wastewaters often fails to conform to the prescribed limits recommended by the pollution control bodies of India (CPCB, 2013). Therefore, we have chosen this site for the study. The collected samples were immediately processed for physico-

chemical parameters analysis, residual organic pollutants detection and charactertization as well as toxicity evaluation tests. The analysed parameters included pH, BOD, COD, TDS, TSS, total chloride, phenolics, nitrate, phosphate and sulfate (APHA, 2012). The pH was measured with a digital pH meter (Metrohm, USA). Digested samples (100mL) in a digestion mixture of nitric-perchloric acid (5:1) were used to determine total chromium using atomic absorption spectroscopy (AAS) (GBC, Avanta Sigma, Australia) (APHA, 2012).

(APHA, 2012).

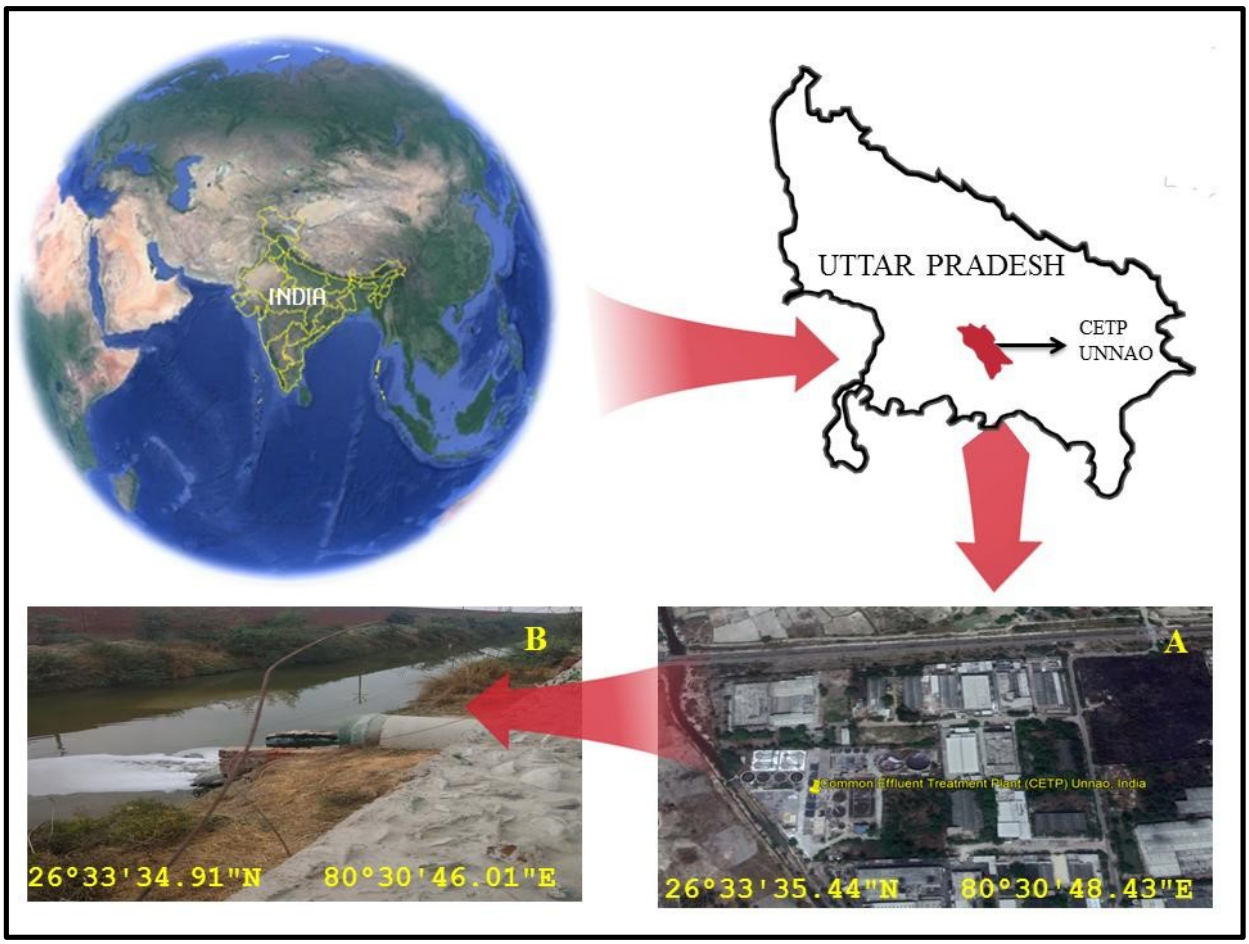


Figure 1: Location of sampling site (CETP, Unnao) and discharge of treated tannery wastewater through a drain into the environment.

2.2. Characterization and identification of residual organic pollutants from treated tannery wastewater

2.2.1. Extraction of residual organic pollutants from collected tannery wastewater by using different solvents systems

Extraction of the residual organic pollutants from wastewater was performed by liquid-liquid extraction (LLE) method using different combination of organic solvents (Minuti et al., 2006). Briefly, centrifuged (8000×*g* for 20minat 4 °C) samples (200mL) were acidified to pH2.0 using 1N HCl and then extracted three times with the equal volume of solvent system containing 100mL of dichloromethane (DCM) and 100mL of diethyl ether (solvent system-1) and 100mL of dichloromethane (DCM) and 100mL of n-haxane (solvent system-2) in a separating funnel (500mL) by intermittent shaking (Haq et al., 2017). The solvent layer containing residual organic pollutants was separated and evaporated to dryness under vacuum at 40 °C. The extracts were dissolved in 2mL of DCM and passed through syringe filter (0.22 µ m). All the organic solvents used were of HPLC grade (purity >99%).

2.2.2. GC-MS/MS analysis

The extracts were derivatized using trimethyl silyl (BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) TMCS) at 60 °C for 15min (Marco et al., 2007). Silylated samples (1 µL) were injected into the GC-MS (PerkinElmer, Waltham, MA, USA) equipped with a PE auto system XL gas chromatograph interfaced with a Turbomass mass spectrometric mass selective detector. Helium gas was used as carrier gas with flow rate of 1mLmin^{-1} in column, which was programmed as: 50 °C (5 min); 50–300 °C (10 °C.min^{-1}, hold time: 5 min). The electron ionization (EI) mass spectrum was recorded in full-scan mode in the range of 30–550 (*m/z*) at 70 eV. To identify the compounds, the mass spectra of peaks were compared with that of National Institute of Standards and Technology (NIST) library available with the equipment.

2.3. Toxicological evaluation of CETP treated tannery wastewater for environmental safety

2.3.1. Phytotoxicity test using *Vigna radiata* L.

Mung bean (*Vigna radiata* L.) seed germination and seedling growth test was performed as per the established protocol (OECD, 2003; Bharagava and Chandra, 2010). Mung bean seeds were purchased from a local certified seed seller shop and healthy seeds were surface sterilized with 0.2% (*w/v*) HgCl2. Six test solutions of treated wastewaters were prepared (6.25%, 12.5%, 25%, 50%, 75% and 100%, *v/v*) with distilled water. Petri dishes (20mm×120mm) containing 10 seeds were irrigated with 5mL test solutions and seeds irrigated with tap water were treated as control. The test Petri dishes were incubation at 28±1 °C in a BOD incubator. The number of seeds germinated was recorded after 48 h and expressed in terms of percentage (%) germination. Seedlings growth parameters (root and shoot length) were measured after 5 days of treatment (Bharagava and Chandra, 2010; Kumari et al., 2014). The studies were experimented in triplicate.

2.3.2. Phytotoxicity test using *Allium cepa* L.

Onion (*Allium cepa* L.*)* root growth inhibition test was carried out by growing the onion bulbs in tannery wastewaters. The onion bulbs purchased from a local market of Lucknow were healthy and equal-sized. The outer layer of onion bulbs and the dry bottom plate was removed, while taking care of root primordial.

Five onion bulbs were placed over 50mL Falcon tubes filled with test solutions (6.25%, 12.5%, 25%, 50% and 100%, v/v). The tubes were kept in an incubator at 23 °C for 5 days. The test solutions stored at 4 °C were refilled morning and evening to ensure the contact between onion bulbs and samples present in the tubes. After 5 days, the onion bulbs exposed at each concentration were observed for the root growth and lengths. The inhibition in root growth was measured and was correlated with an index of degree of toxicity (Fiskesjo, 1985).

2.3.3. Genotoxicity studies

The genotoxicity of treated wastewaters were measured in terms of chromosomal aberrations in the root tip cells of *A. cepa*. This study was conducted using three test solutions (6.25%, 12.5% and 25% v/v) of treated wastewaters. Five onion bulbs were initially rooted in tap water for 48 h till root length reached 1–2cm and then transferred to test solutions for 24 h, to complete the cell cycle in meristematic cells of *A. cepa* roots within 24 h. To evaluate the cyto and genotoxicity, the root tips were fixed in alcohol and glacial acetic acid (3: 1) fixative for 12 h at 60–70 °C and after washing with distilled water, the root tips were hydrolyzed with 1N HCl at 60–70 °C for 5 min. After proper washing the root tips were processed for slide preparation using haemotoxylin as the stain (Chauhan and Sundararaman, 1990; Haq et al., 2017). Mitotic Index was determined by scoring approximately 4000 cells (500–1000 cells per slide). All the slides were microscopically analysed to calculate the mitotic index (MI) and chromosomal aberrations (CA). MI and CAs were calculated using following formula:

Mitotic Index (%) = (Number of dividing cells/Number of total observed cells) ×100.

Chromosomal Aberrations (%) = (Total aberrant cells/Number of total observed cells) ×100.

2.3.4. Statistical data analysis

All the experiments were carried out in triplicates and values are presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) followed by Dunnett's post multiple comparison tests was also performed for MI and CA values. The value of $p<0.05$ was considered significant. Statistical analysis was performed using IBM SPSS Statistics-20.0 software.

3. Results and Discussion

3.1. Physico-chemical characteristics of treated tannery wastewater

Tannery is one of the highest environmental polluting industry due to the discharge of wastewater containing high concentration of hazardous waste including heavy metal like chromium. Over the years,

many new chemicals has been introduced in tanning processes. Hence, detail analysis of wastewater generated from tanneries are essential for better understanding of the toxicity and chemical nature of the effluents. The results of physico-chemical analysis of the CETP treated tannery wastewater is summarized in Table 1. The wastewater was found to have high concentration of BOD (680.00±20mg.L^{-1}), COD (1300.00±45mg.L^{-1}), EC (4.40±0.2MS.cm^{-1}), TDS (3850.00±10.0mg.L^{-1}), (566.00±12.5mg.L^{-1}), sulfate (8.64±0.42mg.L^{-1}), phosphate (26±2mg.L^{-1}), nitrate (12.3±0.3mg.L^{-1}), chloride (1434±12mg.L^{-1}) and phenolic (10.5±0.5m g.L^{-1}). The nature of wastewater was alkaline (pH 8.45±0.18). High concentration of total chromium (7.39±0.06mg.L^{-1}) was also observed. The values of the physico-chemical parameters were higher than the permissible limits for effluent discharge as suggested by CPCB (CPCB, 2013 and USEPA (U.S. Environmental Protection Agency (USEPA), 2004) and Table 1 clearly indicates the toxic nature of wastewater treated by the CETP-Unnao.

The discharge of wastewater from CETP affects the flora and fauna of the aquatic ecosystem by blocking the sunlight penetration in receiving water bodies and photosynthetic activity, thus, negatively affects the aquatic life (Sukumaran et al., 2008; Deepa et al., 2011). The high TDS value is also toxic to aquatic lives by causing osmotic stress and affecting the osmoregulatory functions of organisms (Thakur and Srivastava, 2011). The high BOD and COD values of the tannery wastewater might be due to the presence of high organic contents and salts in the wastewater (Mishra and Bharagava, 2016). High salts are responsible for acidification, reduced soil fertility and increased salinity of groundwater and rivers. The high sulfate, phosphate and nitrate content in the tannery wastewater could be associated with the use of sulfuric acid and sulfide in dehairing process during the tanning process (Yadav et al., 2016a). The high TDS level in wastewater directly indicates the presence of metal ions in the system. Monosodium, disodium phosphates, polyphosphates used in leather treatment processes and ammonium salts in deliming and bating process, are responsible for eutrophication that disturbs the normal ecological functioning of receiving water bodies (Saxena et al., 2016). Phenolics, listed as the “priority pollutant” by the Environmental Protection Agency (USEPA) (2014) owing to its toxic, genotoxic and carcinogenic

effects in plants, animals and human beings, are also high in wastewater due to its utilization in the preservation of raw hides/skins and leather finishing (Mishra and Bharagava, 2016). Chromium used in leather tanning as fastening agent for marking and surfacing of leather was also found to be in higher (7.39mg L^{-1}) (Lofrano et al., 2013; Yadav et al., 2016b) than the permissible limit (2mg L^{-1}). High Cr level causes toxic, genotoxic, mutagenic, and carcinogenic effects on humans, animals, plants, and microbes as reported by various authors (Mishra and Bharagava, 2016; Chowdhary et al., 2018).

Table 1: Physico-chemical characteristics of CETP treated tannery wastewater with reference to the national and international standards.

Parameters	Collected wastewater values	Effluent standards (CPCB, 2013) (U.S. Environmental Protection Agency (USEPA), 2004)	
Color	Light yellowish	-	-
Temperature	32 °C	40 °C	-
pH	8.45±0.18	5.5–9.0	-
EC (Ms/cm)	4.4±0.2	0.4	-
BOD (mgl^{-1})	680.00±20.0	30.00	40.00
COD (mgl^{-1})	1300.00±10.0	250.00	120.00
TS (mgl^{-1})	4416.00±14.0	2200.00	-
TDS (mgl^{-1})	3850.00±10.0	2100.00	-
TSS (mgl^{-1})	566.00±12.5	100.00	-
Sulfate (mgl^{-1})	8.64±0.42	5	-
Chloride(mgl^{-1})	1434.00±12	600.00	-
Phosphate (mgl^{-1})	12.5.00±0.5	5	-
Nitrate (mgl^{-1})	12.3±0.3	10.00	-
Phenolics (mgl^{-1})	10.5±0.5	1–5	0.50
Heavy metal concentration			
Chromium (mgl^{-1})	7.39±0.03	2	0.05

All the values are mean of triplicates (n = 3) ± SD; BOD: Biological oxygen demand; COD: Chemical oxygen demand; TS: Total solids; TDS: Total dissolved solids; TSS: Total suspended solids

3.2. Residual organic pollutants (ROPs) present in treated tannery wastewater

Tannery wastewater has complex mixture of various chemicals pollutants, they are not possible to extract using single solvent extraction method. Therefore, combinations of different solvent systems were used to extract the majority of organic pollutants for GC-MS analysis. The ROPs identified by GC-MS in the extract of DCM-diethyl ether (1:1) using NIST library were mainly derivatives of fatty acids and organic acids (Fig. 2A). In the GC-MS analysis, various major peaks were observed and ROPs were identified at different retention time (RT) viz., RT 6.11 (hexadecanoic acid), 9.77 (Docosane), 12.26 (benzoic acid), 12.83 (3-[4,-(T-Butyl) Phenyl] furan-2-5-dione), 18.44 (benzeneacetamide), 27.44 (resorcinol), 30.35 (dibutyl phthalate), 33.93 (benzene 1,2,4 triol), and 35.52 (1-phenyl-2-phenylthio), respectively. Minor peaks at RT 13.24 (1-pentene1,3-diphenyl), 21.67 (2-bromotetradecanoic acid), 26.67 (phosphoric acid), 37.01 (9-octadecanoic acid), 44.21 (octadecanoic acid) and 48.63 (monopalmitin), respectively.

Further, GC-MS analysis of extracts of DCM-n-hexane (1:1) solvent system showed the presence of fatty acids and carboxylic acids (Fig. 2B & Table 2). The major peaks at RT 7.53 (propanoic acid), 7.93 (banzeneacetic acid), 12.29 (benzoic acid), 12.84 (3-[4,-(T-Butyl) Phenyl]furan-2-5-dione), 13.89 (2-pentenoicacid), 18.44 (benzeneacetamide), 24.07 (dodecanoic acid), 27.44 (resorcinol), 30.34 (dibutyl phthalate), 33.93 (benzene 1,2,4 triol) and 35.52 (1-phenyl-2-phenylthio) were the identified compounds . Compounds at minor peaks at RT 37.01, (9-octadecanoic acid), 41.41 (10-undecynoic acid), 46.47 (Docosanoic acid 1,2,3-propanetriyl) and 48.89 (acetic acid) were also identified.

Table 2: Residual organic pollutants identified as TMS (Trimethylsilyl) derivatives by GC-MS/MS analysis of CETP treated tannery wastewater extracted with solvent system containing dichloromethane + diethyl either (A) and dichloromethane + n-hexane (B).

Retention time (min)	Molecular formula	Identified residual organic compounds	A	B
6.11	$C_{19}H_{40}O_2$	Hexadecanoic acid	+	-
7.53	$C_9H_{20}O_2$	Propanoic acid	-	+
7.93	$C_8H_8O_2$	Benzeneacetic acid	-	+
9.77	$C_{22}H_{46}$	Docosane	+	-
12.26	$C_{10}H_{14}O_2$	Benzoic acid	+	+
12.83	$C_{14}H_{14}O_3P$	3-[4-(T-Butyl)Phenyl]furan-2-5-dione	+	+
13.24	$C_{20}H_{26}O$	1-pentene,1,3-diphenyl	+	-
13.89	$C_{18}H_{38}O_3$	2-pentenoic acid	-	+
18.44	C_6H_9NO	Benzeneacetamide	+	+
21.67	$C_{14}H_{27}BrO_2$	2-Bromotetradecanoic acid	+	-
24.07	$C_{12}H_{24}O_2$	Dodecanoic acid	-	+
26.97	H_3PO_4	Phosphoric acid	+	-
27.44	$C_{12}H_{22}O_2$	Resorcinol	+	+
30.34	$C_{16}H_{22}O_4$	Dibutyl phthalate	+	+
33.93	$C_{15}H_{30}O_3$	Benzene 1,2,4 triol	+	+
35.52	$C_{12}H_{32}O_2$	1-Phenyl-2-phenylthio	+	+
37.01	$C_{18}H_{34}O_2$	9-octadecanoic acid	+	+
44.21	$C_{18}H_{36}O_2$	Octadecanoic acid	+	-
41.41	$C_{12}H_{32}O_2$	10-undecynoic acid	-	+
46.47	$C_{69}H_{134}O_6$	Docosanoic acid, 1,2,3-propanetriyl	-	+
48.63	$C_{25}H_{54}O_4P$	Monopalmitin	+	-
48.89	$C_{19}H_{38}O_2$	Acetic acid	-	+

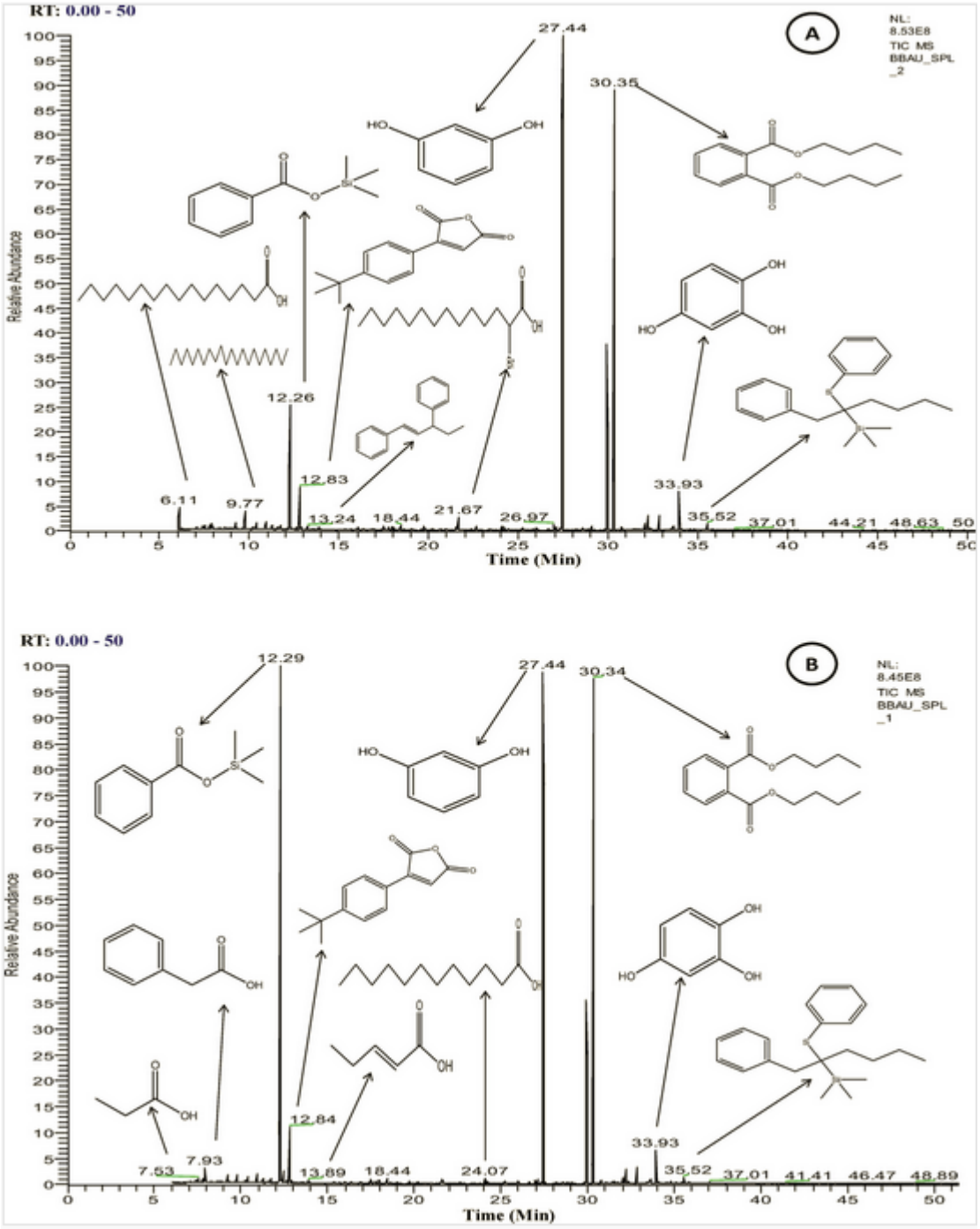


Figure 2: GC-MS chromatogram of dichloromethane + diethyl either (A) and dichloromethane + n-hexane (B) extracts of CETP treated TWW showing the presence of various residual organic pollutants (ROPs).

The detection of compounds in the treated tannery wastewaters clearly indicates (Table 2) the recalcitrant nature of these compounds as they were not degraded completely during the secondary treatment in the CETP and were discharged into the environment along with the wastewater (Chandra et al., 2011). Fatty acids such as hexadecanoic acid, dodecanoic acid, and octadecanoic acid might be originated as a result of processing of raw hide/skins (Saxena et al., 2016). Phthalate (such as dibutyl phthalate: DBP), benzoic acid and resorcinol are used as plasticizers to increase the flexibility and pliability of leather products, as biocide in raw hide/skins preservation and as surfactants, respectively in Phthalates such as, dibutyl phthalate, diethyl phthalate, butylbenzyl phthalate, and diethylhexyl phthalate and phthalic acid have been listed as priority pollutants by USEPA (He et al., 2015). Discharge of these phthalates causes water pollution and serious toxicological effect in aquatic organisms, such as animals and fishes etc (Alam et al., 2009; Chen et al., 2014; Saxena et al., 2016). Benli et al. (2016) also reported that phthalates bioaccumulation leads to genotoxic effects, endocrine disruption, disruption of antioxidant defence system in plants and human.

Phenolic compounds and phthalates are reported as potential endocrine disrupting chemicals (EDCs). Benzoic acid, a well-known EDCs was detected in the tannery wastewater by GC-MS. It has been classified as a Group B2, a probable human carcinogen and highly toxic to aquatic organisms (Kumari et al., 2016; (USEPA, 2012). Benzene is known carcinogen was also observed and its presence in tannery wastewater might be associated with the use of phthalate and phenolics compounds in leather industries (Lyche et al., 2009; U.S. Environmental Protection Agency (USEPA), 2004; Dixit et al., 2015) (Table 2). Further, recently classified EDCs such as resorcinol, hexadecanoic acid, and octadecanoic acid were also detected in tannery wastewater (U.S. Environmental Protection Agency (USEPA), 2004). The result of the present studies suggests that combination of DCM + n-hexane organic solvents was able to extract maximum number of ROPs and thus, will be useful in the extraction of organic pollutants from tannery and other wastewater. In the last few years, leather-tanning has adapted an eco-friendly, non/less toxic and biodegradable chemicals as per strict regulations to limit the pollution level.

However, the quality of treated wastewater has not yet improved, which is apparent from present study.

3.3. Phytotoxicity of tannery wastewater

The toxicity tests combined with physico-chemical analysis are essential in the evaluation of effluent quality. Hence, treated wastewater was assessed for its phytotoxic and genotoxic nature. The seed germination test is considered as one of the simplest short-term, sensitive and cost effective method of phytotoxicity evaluation for wastewaters (Rusan et al., 2015; Lyu et al., 2018). Seed germination is a very sensitive process, likely to be disturbed by the substances present in the environment. In the present study, mung bean (*Vigna radiata L.*) seeds germination test (48 h) was carried out in different concentrations of treated tannery wastewater. The result of mung seed germination inhibition upon exposure to different concentrations of wastewater is given in Table 4, which showed 50% seed germination inhibition at 50% wastewater concentration. Hence, the noted value of IC50% for seed germination was 50% (v/v) concentration (data not shown). At 75 and 100% (v/v) wastewater concentrations, the percentage of seed germination was 10 and 30%, respectively.

The effect of wastewater on early seeding growth (5-seedling) after 5 days is apparent in Table 3. Seedling growths gradually decreased with increasing concentration of treated wastewaters. However, compared to the controls, the root lengths of seedling were highest at 12.5% (v/v) and thereafter gradually decreased with increasing concentrations of treated wastewater (Table 3). Notable reduction in root length, and shoot length were observed at 75 and 100% wastewater concentrations, respectively, which might be due to the effect of high salts and phenolics and ROPs present in the treated wastewater (Kumari et al., 2016). Oliveira (2012) reported that the inhibition of seed germination percentage was associated with high TDS and Cr ion in wastewater causing the osmotic stress and toxicity in plants (Kasoobi, 2017). Phenolics content in wastewater alters the homeostatic of

plants through the over production of reactive oxygen species as reported earlier (William et al., 2017; Lyu et al., 2018).

Further, the phytotoxic effect of tannery wastewater was measured in term of root growth inhibition test using *Allium cepa*. Root growth inhibition in *Allium cepa* root has been considered as a toxicity indicator since it may result from inhibition of the cell division (Fiskesjo, 1985; Egito et al., 2007). The effect of different concentrations of tannery wastewater on root growth and length of *A. cepa* Fig. 3(a) and (b). Initially, the onion bulbs were rooted in different concentrations of wastewater (0–100% v/v) to observe the root growth of *A. cepa* and results showed that wastewater beyond 25% was inhibitory for root growth. The inhibition was more pronounced at 50%. The IC50% value of wastewater for root growth inhibition was 10% (v/v) wastewater concentration (data not shown). The recorded mean root lengths after 5 days treatment were 0.6, 2.2, 5.6, and 7.1cm when grown in 50%, 25%, 12.5%, 6.25% and 0%, respectively (Fig. 3b). The prevention of root growth above 25% water concentration is indicative of the presence toxic pollutants in tannery wastewater. The chromium and GC-MS detected other residual organic pollutants such as benzoic acid, 3-[4,-(T-butyl) Phenyl] furan-2-5-dione, benzeneacetamide, resorcinol, dibutyl phthalate, benzene-1,2,4-triol, and 1-Phenyl-2-phenylthio detected in tannery wastewater are earlier reported to cause cell division, change in chlorophyll contents, which directly influences the root growth, length and biomass of plant (Salminen and Karonen, 2011; Gao and Wen, 2016; Lyu et al., 2018).

Table 3: Effect of different concentrations of CETP treated tannery wastewater on seed germination, root length, and shoot length in mung bean (*Vigna Radiata*) plant**.**

Wastewater (%)	Germination (%)	Root length (cm)	Shoot length (cm)
0	100±0.0	1.5±0.3	5.7±0.2
6.25	100±0.0	0.9±0.1	4.6±0.2
12.5	90±0.5	0.5±0.1*	3.1±0.2*
25	70±0.5	0.37±0.05*	1.5±0.5*
50	50±0.9	0.06±0.6*	0.8±0.1*
75	0±0.0	0±0.0*	0±0.0*
100	0±0.0	0±0.0*	0±0.0*

Values are mean ± SD (n=3). The *refers to statistically significant difference from control (p<0.05)

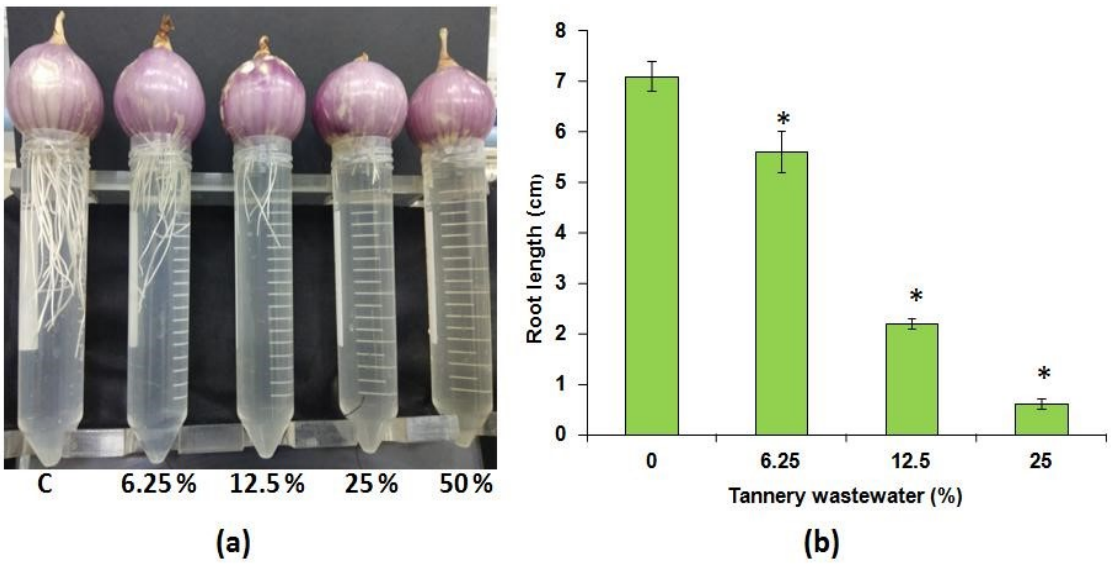


Fig 3: Effect of different concentrations of CETP-treated tannery wastewater on root growth (a) and root length (b) of *A. cepa*. Values are mean ± SD of three samples. $p < 0.05$, significant when compared to control using ANOVA.

3.4. Cytotoxicity and genotoxicity of tannery wastewater

The cytotoxic and genotoxic effects of treated tannery wastewater were evaluated on the basis of mitotic index (MI) and chromosomal aberrations (CA) in root tip cell of *A. cepa*.

3.4.1. Mitotic index

Mitotic index (MI) is a good experimental method to assess the cytotoxic effect of variety of pollutants in the cell division. MI measures the proportion of cells in mitotic phase of a cell cycle and its inhibition could be interpreted as cellular death (Rojas et al., 1993). The cytotoxic effect of treated tannery wastewater in *A. cepa* roots is summarized in Table-4. Results revealed that the percent mitotic index (MI%) value of plant root was in the order of 31%, 23%, and 16% as compared to control (66%) at the concentration of 6.25, 12.5%, and 25% wastewater respectively. The MI% decreased progressively with increasing wastewater concentrations indicating the presence of various cytotoxic residual organic pollutants in the treated tannery wastewaters. These pollutants may interfere with normal process of mitosis, thus preventing a number of cells from entering the prophase and blocking the mitosis cycle during interphase (Srivastava, 2015; Haq et al., 2017). The inhibition of MI% may be attributed to the effect of pollutants on DNA/protein synthesis. The results are in agreement with earlier studies where *A. cepa* root cells were exposed to wastewaters (Rojas et al., 1993; Haq et al., 2017).

Table 4: Effect of different concentrations of CETP treated tannery wastewater on Mitotic index (%) of root tip cells of *A. cepa*.

Exposure (24 h)		Total cells	Dividing cells	MI%
Water	Control	570	375	66±7.0
Wastewater	6.25%	542	170	31±8.0*
	12.5%	590	135	23±1.0*
	25%	506	80	16±2.0*

Values are mean ± SD (n=3). The *refers to statistically significant difference from control ($p<0.05$), TWW = Tannery wastewater

3.4.2. Chromosomal aberrations

Chromosomal aberration (CA) analysis of the root tip cells of *A. cepa* is considered as an efficient test to investigate the genotoxic, clastogenic and aneugenic potential of chemical agents and industrial wastewaters. CA has been characterized by changes in either of chromosomes structure, which can occur both spontaneously and as well as result of the exposure to physical or chemical agents (Kumari et al., 2016; Papa et al., 2016). Various types of chromosomal aberrations are considered over the four stages of the cell cycle (prophase, metaphase, anaphase, and telophase) as depicted in Table 5 and Fig. 4.

Results showed that there was no chromosomal abnormalities in the control cells treated with tap water. On the other hand, treatment with different concentrations of tannery wastewater induced various types of chromosomal aberrations and nuclear abnormalities (Fig. 4). The observed aberrations were chromosome loss (Fig. 4a), vagrant chromosome (i.e. moving/wondering chromosomes having no defined place) (Fig. 4b), sticky metaphase (i.e. clumping of chromosomes in metaphase stage) (Fig.4c), c-mitosis (i.e. induced abortive nuclear division leading to the doubling in chromosome numbers) (Fig. 4d), binucleated (i.e. cell having two nuclei) (Fig. 4e) and micronuclei (i.e. small nucleus formed whenever a chromosome or a fragment of a chromosome is not incorporated into one of the daughter nuclei during

cell division) (Fig. 4f). The most frequent aberrations were c-mitosis, vagrant, and stickiness chromosomes at all the tested wastewaters concentration.

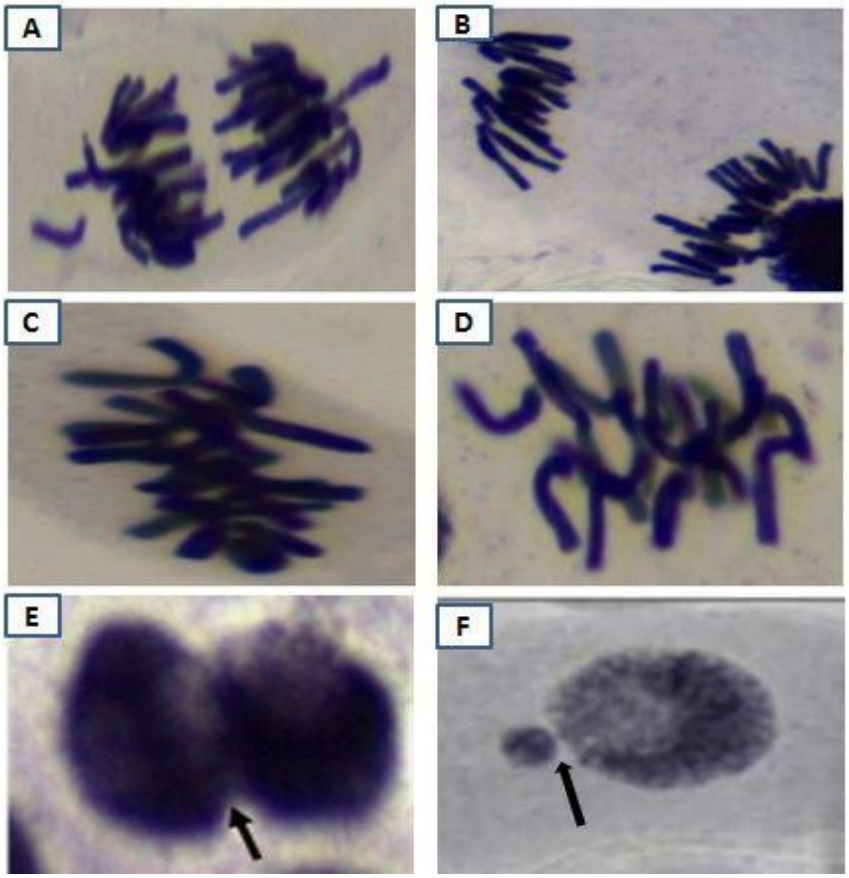


Fig. 4: Chromosomal aberrations observed in root tip cells of *A. cepa* exposed with different concentrations of CETP treated tannery wastewater. (a) chromosome loss, (b) vagrant chromosome, (c) Sticky metaphase, (d) c-mitosis (e) Binucleated (f) Micronucleated.

The percentage of aberrant cells was concentration-dependent and it was the highest (38.3%) at 25% wastewater concentration. The induction of various chromosomal aberrations in the root tip cells of *A. cepa* was possibly due to the presence of residual organic pollutants as detected by GC-MS and heavy metals including chromium (Kumari et al., 2016).

Table 5: Different chromosomal and nuclear abnormalities observed in root tip cells of *A. cepa* exposed with different concentrations of CETP treated tannery wastewater.

Assay	Water	Tannery wastewater		
		6.25%	12.5%	25%
Chromosomal aberrations				
Stickiness	0.0±0.0	2.3±0.5	5.0±1.0	7.0±1.0
Vagrant	0.0±0.0	3.0±1.0	3.0±1.0	4.3±1.5
Chromosomal loss	0.0±0.0	2.0±1.0	2.7±0.6	4.3±0.6
C-mitosis	0.0±0.0	2.7±1.5	3.3±1.5	4.3±1.5
Binucleated	0.0±0.0	2.3±0.6	4.0±1.0	5.3±1.5
Micronuclei	0.0±0.0	3.0±1.0	3.0±1.0	5.3±1.5
Aberrant cells (%)	0.0±0.0	9.0±1.2*	15±1.7*	38.3±2.0*

Values are mean ± SD (n=3). Chromosomal aberrations were scored on 500–1000 cells per slide. The *refers to statistically significant difference from control ($p<0.05$), TWW= Tannery wastewater

Stickiness is considered a common sign of toxic effects on chromosomes probably leading to cell death (Rojas et al., 1993). Stickiness of chromosomes may also occur due to either increased chromosomal contraction and condensation or depolymerization of DNA and partial dissolution of nucleoproteins (Turkoglu, 2007). The occurrence of chromosomal loss and vagrant chromosomes suggests spindle failure (Haq et al., 2017). Colchicine mitosis (c-mitosis) is defined as the inactivation of spindle followed by random scattering of chromosomes around the cells. The wastewater induced a high frequency of c-mitosis, which has been also shown by other studies indicating that wastewater is comparable toxic to colchicine and thus capable to induce C-mitosis.

Conclusions

The Residual organic pollutants and toxicity characterization studied of CETP treated tannery reveals toxic nature of wastewater with the following observations:

- CETP treated wastewater was found to have very high BOD, COD, TDS, sulfate and phenolics which are above the prescribed limits.
- The wastewater contained high level of toxic chromium (7.39mg.L-1) and a variety of residual organic pollutants such asbenzoic acid, 3-[4,-(T-butyl) Phenyl] furan-2-5-dione, benzeneacetamide, resorcinol, dibutyl phthalate, benzene-1,2,4-triol, and 1-Phenyl-2-phenylthio.
- The wastewater was phytotoxic, as it inhibited seed germination, root and shoot *in V. radiata* upon exposure to diluted samples.
- It inhibited mitotic index and induced chromosomal aberration in root tip cells of *A. cepa.* The observed chromosomal aberration in root tip cells of *A. cepa* were stickiness, chromosome loss, vagrant chromosome and C-mitosis.

This study indicated that there is a need to adopt a proper treatment and bioremediation strategies to reduce the pollution load of tannery wastewater for the safety of the environment.

Conflict of interest

The authors declare that they have no conflict of interest.

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